Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast

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Although programmed cell death (PCD) is extensively studied in multicellular organisms, in recent years it has been shown that a unicellular organism, yeast *Saccharomyces cerevisiae*, also possesses death program(s). In particular, we have found that a high doses of yeast pheromone is a natural stimulus inducing PCD. Here, we show that the death cascades triggered by pheromone and by a drug amiodarone are very similar. We focused on the role of mitochondria during the pheromone/amiodarone-induced PCD. For the first time, a functional chain of the mitochondria-related events required for a particular case of yeast PCD has been revealed: an enhancement of mitochondrial respiration and of its energy coupling, a strong increase of mitochondrial membrane potential, both events triggered by the rise of cytoplasmic $[Ca^{2+}]$, a burst in generation of reactive oxygen species in center *o* of the respiratory chain complex III, mitochondrial thread-grain transition, and cytochrome *c* release from mitochondria. A novel mitochondrial protein required for thread-grain transition is identified.

Introduction

Apoptotic cell death is a key event during the development of multicellular organisms. Apoptosis of damaged cells is also a common defense mechanism used throughout animal and plant kingdoms. There is accumulating evidence that unicellular organisms are also equipped with suicide programs (for reviews see Raff, 1998; Lam et al., 1999; Lewis, 2000; Skulachev, 2001, 2002).

Studies on the unicellular yeast *Saccharomyces cerevisiae* are especially promising because it is a genetically tractable organism with a well studied biochemistry. Indications that apoptosis-like programs are present in *S. cerevisiae* were obtained in studies where pro- or antiapoptotic mammalian genes were incorporated into the yeast genome. In particular, Kane et al. (1993) reported that an antiapoptotic mammalian protein, Bcl-2 (β-cell lymphocytic-leukemia protooncoprotein-2), rescued a yeast mutant lacking superoxide dismutase. Additionally, Zha et al. (1996) and Manon et al. (1997) found that proapoptotic mammalian protein Bax caused growth arrest and death of the yeast cells. Several other studies revealed that certain mutations in the *S. cerevisiae* genome cause cell death entailing the appearance of features of the mammalian cell apoptosis (Madeo et al., 1997; Yamaki et al., 2001; Zhang et al., 2003; Qi et al., 2003). Certain harsh treatments induce cell death resembling apoptosis. Madeo et al. (1999) and Ligr et al. (2001) and Ludovico et al. (2001) and Davermann et al. (2002) reported that H_2O_2 and acetic acid, respectively, kill yeast in a process accompanied by typical apoptotic changes. High [NaCl] (Huh et al., 2002), amphotericin B (Phillips et al., 2003), and the DNA-damaging drug adozelesin (Blanchard et al., 2002) were shown to kill yeast, inducing some features of apoptosis. Aging yeast cells were also found to demonstrate multiple apoptotic markers (Laun et al., 2001; Herker et al., 2004; Fabrizio et al., 2004). Furthermore, mutations have been described that seem to extend yeast life span by inactivating the suicide program (Fabrizio et al., 2001, 2003, 2004). Yeast analogues of some components of the apoptotic cascade have been described, i.e., a caspase (Madeo et al., 2002b), Omi (Fahrenkrog et al., 2004), and apoptosis-inducing factor (Wissing et al., 2004; for reviews on the programmed death in yeast see Skulachev, 2002; Jin and Reed, 2002; Madeo et al., 2002a; Breitenbach et al., 2003; Weinberger et al., 2003).

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Abbreviations used in this paper: ΔΨ, mitochondrial transmembrane electric potential difference; FCCP, trifluoromethoxycarbonylcyanide phenylhydrazone; H_2DCF-DA, dichlorofluorescin diacetate; NAC, *N*-acetyl cysteine; PCD, programmed cell death; ROS, reactive oxygen species.

The online version of this article includes supplemental material.

Narasimhan et al. (2001) showed that the plant antibiotic osmotin caused an apoptosis-like death of yeast. Surprisingly, certain components of the pheromone cascade were reported to be involved in the osmotin killing. The reason for this became clear when we described pheromone-induced programmed death of *S. cerevisiae* (Severin and Hyman, 2002). Although low concentrations of the pheromone activate the MAPK cascade and initiate the mating program, higher doses of pheromone induce cell death (for review see Kurjan, 1993). We found that this death shows several traits typical for the mitochondria-dependent apoptosis of animal cells (Severin and Hyman, 2002; Table I).

Importantly, mitochondria are essential for most of the types of apoptosis in animals (Newmeyer et al., 1994; Skulachev, 1999). In particular, it was found that apoptosis-inducing factor (Susin et al., 1996, 1999) and cytochrome *c* (Liu et al., 1996; Yang et al., 1997; Kluck et al., 1997), normally sequestered in the mitochondrial intermembrane space, are released into the cytosol in response to various apoptotic stimuli, activating the apoptotic cascade.

A limitation of studies on the role of mitochondria in the pheromone-induced yeast death arises from the fact that formation of de novo protein(s) is involved (Severin and Hyman, 2002), complicating kinetic analysis of fast stages of the process downstream of translation, at which mitochondria are most probably involved. To overcome such a difficulty, we looked for ways to trigger the same cascade downstream of translation. We reasoned that one way of activating the cell death program could be by disrupting Ca^{2+} homeostasis because even low concentrations of pheromone appear to be toxic for yeast calmodulin mutants (Cyert and Thorner, 1992; Moser et al., 1996). Therefore, we tried several Ca^{2+} ionophores and amiodarone. Although Ca^{2+} ionophores and amiodarone showed qualitatively similar effects, it was amiodarone that was found to be the fastest. In mammals, amiodarone (i.e., 2-butyl-3-benzofuranyl 4-[2-(diethylamino)-ethoxy]-3,5-diiodophenylketone hydrochloride) is known as an antiarrhythmic agent. It blocks the L-type Ca^{2+} channels, Na^+ channels, and β-adrenergic receptors (Varbiro et al., 2003). Recently, it was found to possess broad-range fungicidal activity (Courchesne, 2002), inducing an increase of cytosolic concentration of Ca^{2+} that comes to cytosol from both extracellular media and intracellular depot (Courchesne and Ozturk, 2003; Gupta et al., 2003). Furthermore, it was known that α-factor caused a major increase in cytosolic $[Ca^{2+}]$ (Ohsumi and Anraku, 1985). Our observation that a mutant with a compromised calmodulin-calcineurin system was sensitized to the α-factor–induced death seemed to point to an involvement of Ca^{2+} (Severin and Hyman, 2002).

In the present paper, we show that both high pheromone and amiodarone can trigger the sequential apoptotic changes in mitochondria.

Our data suggest that a Ca^{2+}-induced activation of mitochondrial respiration and mitochondrial transmembrane electric potential difference ($\Delta\Psi$) increase, followed by reactive oxygen species (ROS) production and mitochondrial thread-grain transition, are the key steps in the pheromone-induced programmed death of yeast. A mitochondrial protein (Ysp1), specifically required for the mitochondrial thread-grain transition, de-energization, and the cell death, was identified.

Results

Amiodarone initiates the mitochondria-based steps of the pheromone-linked programmed death cascade

In the first series of experiments, we confirmed the data of Courchesne (2002) that micromolar amiodarone kills *S. cerevisiae* (Fig. 1 A). The data presented in Fig. 1 (B and C) and summarized in Table I reveal striking similarity between the pheromone- and amiodarone-induced deaths. In both cases, an increase in

Table I. **Comparison of the inhibitory patterns of the α-factor and amiodarone effects**

Inhibitor of the death program	Target	Inhibition of death program caused by	
		α-factor	amiodarone
Δ*ste20*	Ste20	+	−
cycloheximide	translation	+	−
rho[0] mutation	mtDNA	+	+
Δ*cyc3*	cytochromes *c*	+	+
myxothiazol	Q_o center	+	+
antimycin A	Q_i center	−	−
KCN	Cyt. ox.	+	+
oligomycin	F_0	+	+
FCCP (0.4 μM)	$\Delta\Psi_m$	+	+
FCCP (6 μM)	$\Delta\Psi_m$	−	−
C-PTIO	NO↓	ND	−
SNP	NO↑	ND	+
NAC	ROS↓	+	+
α-tocopherol	ROS↓	+	+
Δ*ysp1*	Ysp1	+	+
		Stimulation of death program caused by	
Activator of the death program	Target	α-factor	amiodarone
Δ*cmd 1–6*	calmodulin-calcineurin system	+	+

survival is induced by rho^0 mutation, inability to form active cytochrome c ($\Delta cyc3$, a deletion of holocytochrome c synthase), addition of mitochondrial respiratory chain inhibitors (myxothiazol or KCN), H^+-ATP-synthase inhibitor oligomycin, low concentrations of protonophorous uncoupler trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP), antioxidants (N-acetyl cysteine [NAC] or α-tocopherol), and a deletion in the gene $ysp1$ (concerning this mutation, see section Amiodarone-induced Ysp1-dependent fragmentation of mitochondrial filaments). Mutation arresting the calmodulin-calcineurin system sensitized yeast to the killing by both α-factor and amiodarone. Addition of an NO precursor, sodium nitroprusside, increased the survival after the amiodarone treatment, which might be explained by a cytochrome oxidase inhibition by NO. In contrast, a NO scavenger carbonyl phenyl-tetramethylimidazole oxide was without statistically significant effect. As for such a respiratory chain inhibitor as antimycin A, it was without any reproducible influence in both α-factor and amiodarone cases (unpublished data).

To confirm that the amiodarone-induced death, similar to programmed cell death (PCD) triggered by α-factor, is accompanied by apoptotic markers, we checked the localization of cytochrome c and DNA fragmentation after amiodarone addition. As shown in Fig. 1 D, 30 min of amiodarone treatment initiates cytochrome c release from mitochondria to cytosol. DNA fragmentation is also induced as shown by TUNEL and DNA cleavage assays (Fig. 1, E and F). In line with the observations of Courchesne and Ozturk (2003) and Gupta et al. (2003), we observed that amiodarone, similar to pheromone, induces a strong increase in cytosolic $[Ca^{2+}]$. A significant Ca^{2+} increase was found even in the presence of EGTA when only the intracellular Ca^{2+} pools could be its source (Fig. 1 G).

Thus, the amiodarone- and pheromone-induced cell deaths are very similar. Because the effects of amiodarone were stronger and very much faster than those of α-factor, the following analysis was done mainly using the amiodarone system.
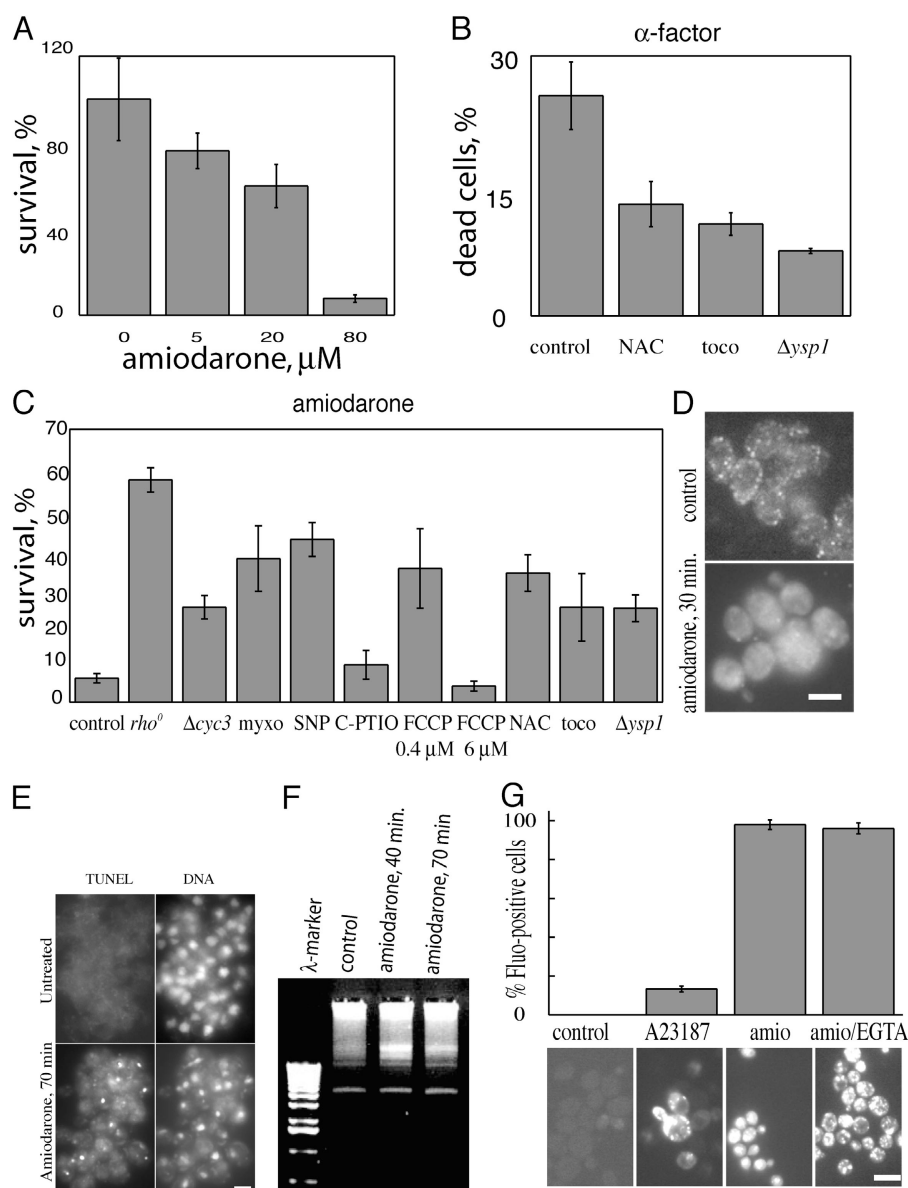
Figure 1. **Pheromone- and amiodarone-induced cell death of yeast share common features, including apoptotic markers.** (A) Amiodarone treatment decreases the percentage of colony-forming cells. (B) 0.1 mg/ml α-factor was added. (C) 80 μM amiodarone was added to all of the samples. Other additions were as follows: 2 μM myxothiazol (myxo); 3 mM sodium nitroprusside (SNP); 1 mM carbonyl phenyl-tetramethylimidazole oxide (C-PTIO); 30 mM NAC; 20 μM α-tocopherol (toco). Cell survival in amiodarone experiments was determined by plating and in pheromone experiments by counting Methylene blue-positive cells. Error bars represent SD of three independent experiments. (D) Cytochrome c is released from mitochondria after 30 min of amiodarone treatment. Fragmentation of mitochondrial filaments in control is an artifact of formaldehyde fixation. (E and F) DNA is fragmented as shown by TUNEL and DNA cleavage, respectively. (G) Amiodarone, similar to Ca^{2+} ionophore A23187, triggers a cytosolic Ca^{2+} rise inside the cells. The effect of 80 μM amiodarone (amio) is relatively stronger as shown by the representative images and the percentages of Fluo-3/AM-positive cells. The effect of amiodarone is not abolished by EGTA and, hence, does not depend critically on the external Ca^{2+}. Bars, 10 μm.

Figure 2. Effect of amiodarone on respiration of *S. cerevisiae* cells or mitochondria. Additions were as follows: (A and B) yeast, 0.6×10^7 cells/ml; 10 µg/ml oligomycin (oligo); 1 µM FCCP; amiodarone (amio); 7 µM myxothiazol (myxo). (C) Yeast mitochondria (0.1 mg of protein/ml; mito); 150 µM ADP; 1 µg/ml oligomycin (oligo); 0.5 µM FCCP; 5 µM amiodarone. Numbers near the curves: nM O_2/min $\times$ 10^{-7} cells (A) or nM O_2/min $\times$ mg protein (C).
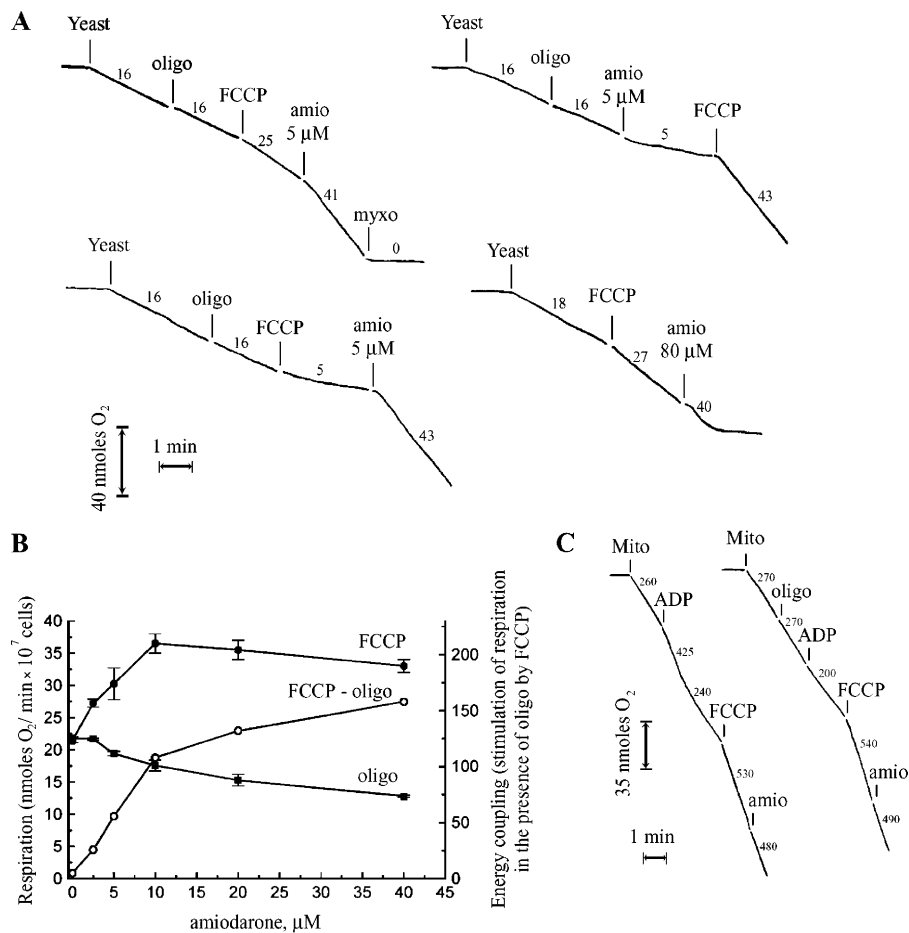
Effects of amiodarone on respiration

As the mitochondrial respiratory chain and energy coupling inhibitors strongly affect the amiodarone-induced death, we have tested the effect of amiodarone on yeast respiration. We found that respiration of *S. cerevisiae* cells growing on galactose and raffinose is relatively slow. Its rate cannot be further lowered by the H^+-ATP-synthase inhibitor oligomycin and is stimulated by uncoupler FCCP (1 µM) only slightly, the effect being transient (Fig. 2 A). Much higher (10 µM) FCCP was necessary to obtain a high and steady respiration rate. Myxothiazol (Fig. 1 A) as well as antimycin A and KCN (not depicted) completely arrested respiration. Addition of low (2–10 µM) concentrations of amiodarone was found to (a) inhibit respiration in the presence of oligomycin and (b) stimulate it in the presence of 1 µM FCCP in a myxothiazol-sensitive fashion (Fig. 2, A and B). High (40–80 µM) amiodarone caused transient stimulation of uncoupled respiration, followed by its strong inhibition (Fig. 2 A). The latter effect can be explained by the fact that some of the respiratory enzymes are arrested by these amounts of amiodarone (see Discussion). As for the low amiodarone, it seems to increase energy coupling and the maximal respiratory rate. The latter should be upstream of cytochrome oxidase because in the myxothiazol-treated cells, uncoupled oxidation of TMPD and ascorbate donating electrons directly to cytochrome oxidase was not stimulated by amiodarone (unpublished data).

It seems likely that a Ca^{2+}-induced activation of an external (noncoupled) NADH dehydrogenase on the outer surface of the inner mitochondrial membrane is a primary reason for amiodarone-induced burst in respiration. Such an activation was described for a homologous enzyme of plant mitochondria (Rasmusson and Moller, 1991). In our hands, addition of Ca^{2+} to isolated yeast mitochondria resulted in a two- to threefold stimulation of oxidation of external NADH, oxidation of pyruvate and malate being unaffected (unpublished data). As for energy coupling increase, it might also be related to cytosolic $[Ca^{2+}]$ rise because in yeast, unlike animals, a nonspecific pore in the inner mitochondrial membrane is inhibited by extramitochondrial Ca^{2+} (Perez-Vazquez et al., 2003). Consistent with this reasoning, all of the effects of low amiodarone on respiration proved to be cell specific. They were absent in isolated yeast mitochondria, which show oligomycin inhibition and FCCP stimulation independently of amiodarone addition (Fig. 2 C). However, the inhibitory action of high amiodarone could also be reproduced on isolated mitochondria if pyruvate and malate (but not external NADH) were used as substrates (unpublished data).

Thus, our data suggest that, without amiodarone, respiration in the actively glycolysing yeast cells used in our experiments was partially uncoupled and its rate was limited by factors other than energy coupling. Addition of amiodarone to the cells shifts control of respiration from the substrate dehydrogenases or respiratory chain enzymes to the level of energy coupling.
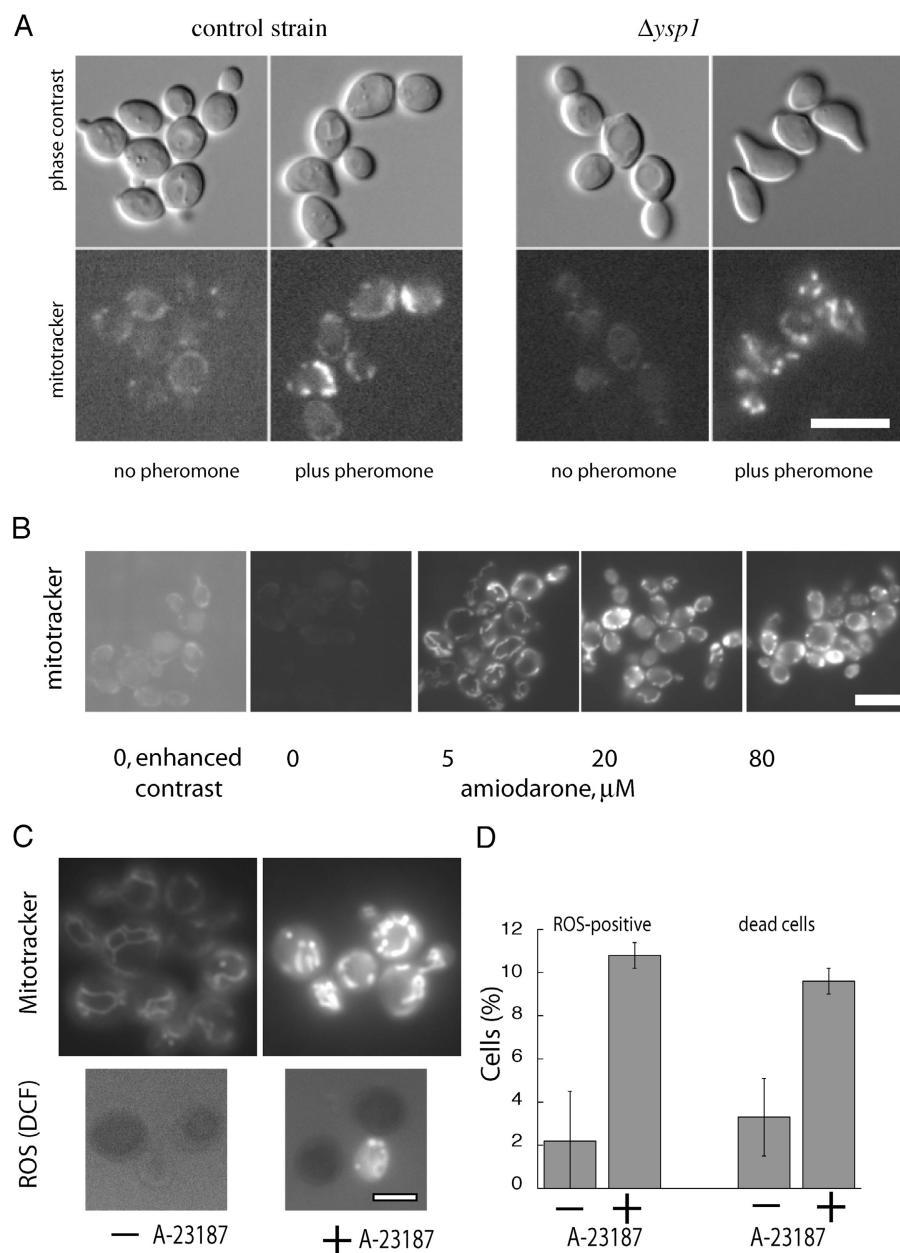
A

control strain

$\Delta ysp1$

phase contrast

mitotracker

no pheromone   plus pheromone

no pheromone   plus pheromone

B

mitotracker

0, enhanced
contrast

0

5

20

80

amiodarone, µM

C

Mitotracker

ROS (DCF)

— A-23187

+ A-23187

D

ROS-positive   dead cells

Cells (%)

— +
A-23187

— +
A-23187

Figure 3. **The pheromone- and amiodarone-induced increase in mitochondrial membrane potential in the yeast cells is Ca²⁺ mediated.** Mitotracker orange was used for the $\Delta\Psi$-dependent staining of mitochondria. (A) 0.1 mg/ml of the pheromone (α-factor). (B) Numbers below panels show amiodarone concentrations (µM). (C and D) Ca²⁺ ionophore A23187 triggers $\Delta\Psi$ increase, ROS accumulation (C), and cell death (D). Error bars represent SD of three independent experiments. Bars, 10 µm.

An increase in membrane potential and ROS production during amiodarone-induced PCD

Is respiration in the amiodarone-treated yeast coupled to generation of the mitochondrial membrane potential? Apparently yes, because an uncoupler (1 µM FCCP) discharging $\Delta\Psi$ strongly stimulated this respiration (Fig. 2, A and B). Such a conclusion was confirmed by measuring $\Delta\Psi$ in the yeast cells. To monitor $\Delta\Psi$ enhancement, Mitotracker orange was used. This probe irreversibly stains mitochondria in a $\Delta\Psi$-dependent fashion (the staining is brighter when $\Delta\Psi$ is high). As shown in Fig. 3 B, Mitotracker staining could be imaged without amiodarone at the enhanced contrast only. However, 5 µM, and especially 20 or 80 µM, amiodarone initiated a strong Mitotracker staining. Importantly, 5-min treatment with 80 µM amiodarone is sufficient to induce a $\Delta\Psi$ rise (Fig. 4). A

similar (although slower) $\Delta\Psi$ increase was observed when α-factor was added instead of amiodarone (Fig. 3 A). It was suggested that the abnormally elevated $\Delta\Psi$ directly drives the formation of ROS, and these ROS are the necessary step in development of the apoptotic cascade (Skulachev, 1996a; Korshunov et al., 1997; Davermann et al., 2002). To test this hypothesis, we monitored kinetics of the formation of ROS after amiodarone addition. Similar to pheromone (Severin and Hyman, 2002), amiodarone treatment strongly enhanced the ROS level in the yeast cells (Figs. 4 and 5). Moreover, it was found that the $\Delta\Psi$ increase takes place earlier than the visible ROS accumulation (Fig. 4). Importantly, added antioxidants (NAC or α-tocopherol) drastically increased the survival after the pheromone or amiodarone treatments (Fig. 1 and Table I), indicating that ROS formation is a necessary step in the onset of the cell death process. Again, as in the case of $\Delta\Psi$, the
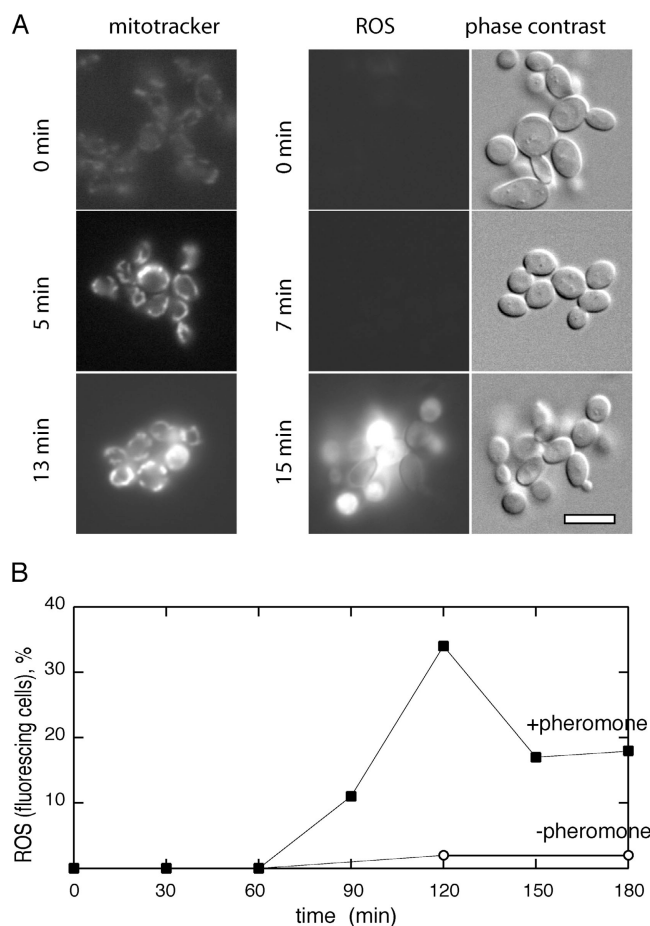
Figure 4. **The time courses of amiodarone- and pheromone-induced increases in ΔΨ and ROS.** (A) ΔΨ and ROS after the addition of 80 μM amiodarone. (B) ROS increase induced by 0.1 mg/ml α-factor. The H₂DCF-DA (50 μM) staining was used as a probe for ROS. Bar, 10 μM.

Figure 5. **Effects of a protonophorous uncoupler of oxidative phosphorylation FCCP on the amiodarone-induced ΔΨ (Mitotracker orange) and ROS (H₂DCF-DA) increases.** Mitotracker orange and H₂DCF-DA were revealed by microscopy (A) and FACS (B). 80 μM amiodarone was added to all samples at time zero. Bar, 10 μm.

pheromone effect on ROS developed much slower than that of amiodarone (Fig. 4, compare A and B). Similar relationships were revealed by others when intracellular Ca^{2+} was monitored. According to Ohsumi and Anraku (1985), the major pheromone-induced rise in $[Ca^{2+}]$ takes 2 h, whereas a similar effect of amiodarone is observed in 1 min (Courchesne and Ozturk, 2003; Gupta et al., 2003).

Is the rise of intracellular Ca^{2+} concentration sufficient to trigger the cell death cascade? To answer this question, we attempted to induce the rise of cytosolic Ca^{2+} by adding ionophore A23187 to the medium. We found that although qualitatively the A23187 effect (monitored by ROS and cell death) was similar to those of pheromone and amiodarone, its rate was rather slow (Fig. 3, C and D). Possibly the relatively weak ROS accumulation and low cell death rate induced by A23187 were due to its low efficiency in increasing cytosolic $[Ca^{2+}]$. Indeed, we found that amiodarone induced much faster intracellular $[Ca^{2+}]$ rise compared with A23187 (Fig. 1 G).

To show that mitochondrial ΔΨ is really required for the ROS formation, we prevented a ΔΨ increase by adding FCCP. As expected, both ΔΨ and ROS increases were abolished. For such effects, 0.4 μM FCCP proved to be sufficient (Fig. 5). Importantly, 0.4 μM FCCP also elevated the cell survival after the
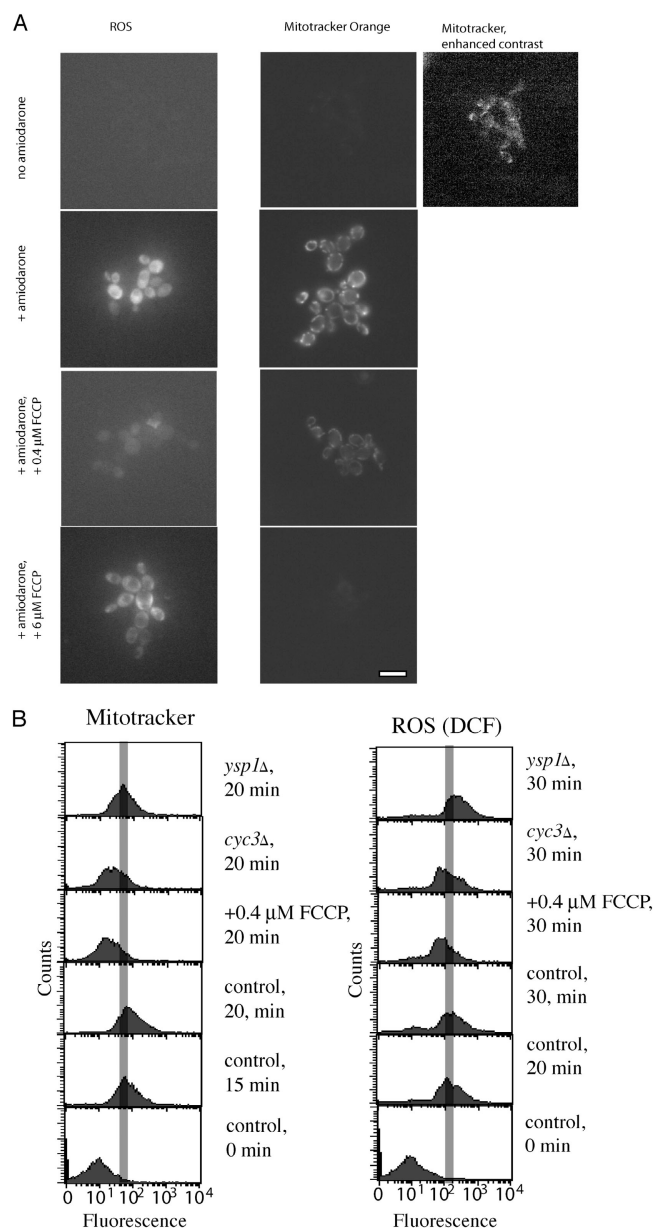
amiodarone treatment (Fig. 1 B), indicating that a ΔΨ increase and related ROS production are not side effects but necessary steps in the cell death cascade.

High (6 μM) FCCP induced an increase in ROS with no ΔΨ observed (Fig. 5), with a negative effect on the survival (Fig. 1 B). The effect is not specific to FCCP because the same was observed when FCCP was replaced by another protonophorous uncoupler, SF6847 (3,5-di(*tret*)butyl-4-hydroxybenzylidene malononitrile; unpublished data).

The low FCCP-sensitive ROS formation might be explained by generation of O_2^+ in the Q-cycle (for discussion, see Fig. 9). To verify this hypothesis, we tested the effects of myxothiazol and antimycin A, two inhibitors that block two
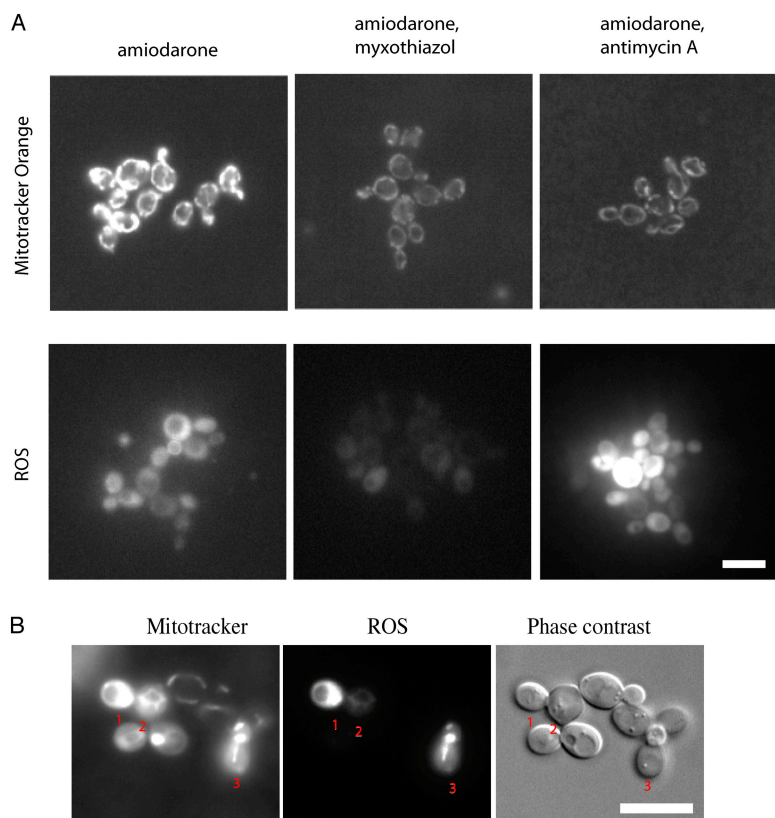
Figure 6. **The cellular effects of amiodarone are mediated by mitochondria.** (A) Effects of the Q-cycle inhibitors myxothiazol (2 μM) and antimycin A (10 μM) on the amiodarone-induced increase in ΔΨ and ROS (Mitotracker orange and H₂DCF-DA, respectively). (B) Colocalization of the Mitotracker orange and H₂DCF-DA staining inside the yeast cell (numbered 1–3). 80 μM amiodarone was always present. Bar, 10 μm.

different stages of the Q-cycle (centers *o* and *i*, respectively). As expected, the inhibitors abolished ΔΨ, oppositely affecting the ROS level. Namely, myxothiazol arrested the ROS increase, whereas antimycin A increased it (Fig. 6 A). Additionally, isolated yeast mitochondria were shown to produce H_2O_2 at rates of 460 nM/min/mg of protein and 130 nM/min/mg of protein with and without antimycin A, respectively. Without antimycin A, FCCP proved to be inhibitory for the H_2O_2 production (unpublished data).

Finally, the colocalization of fluorescent signals of Mitotracker orange and DCF confirmed that mitochondria are responsible for amiodarone-induced ROS production (Fig. 6 B).

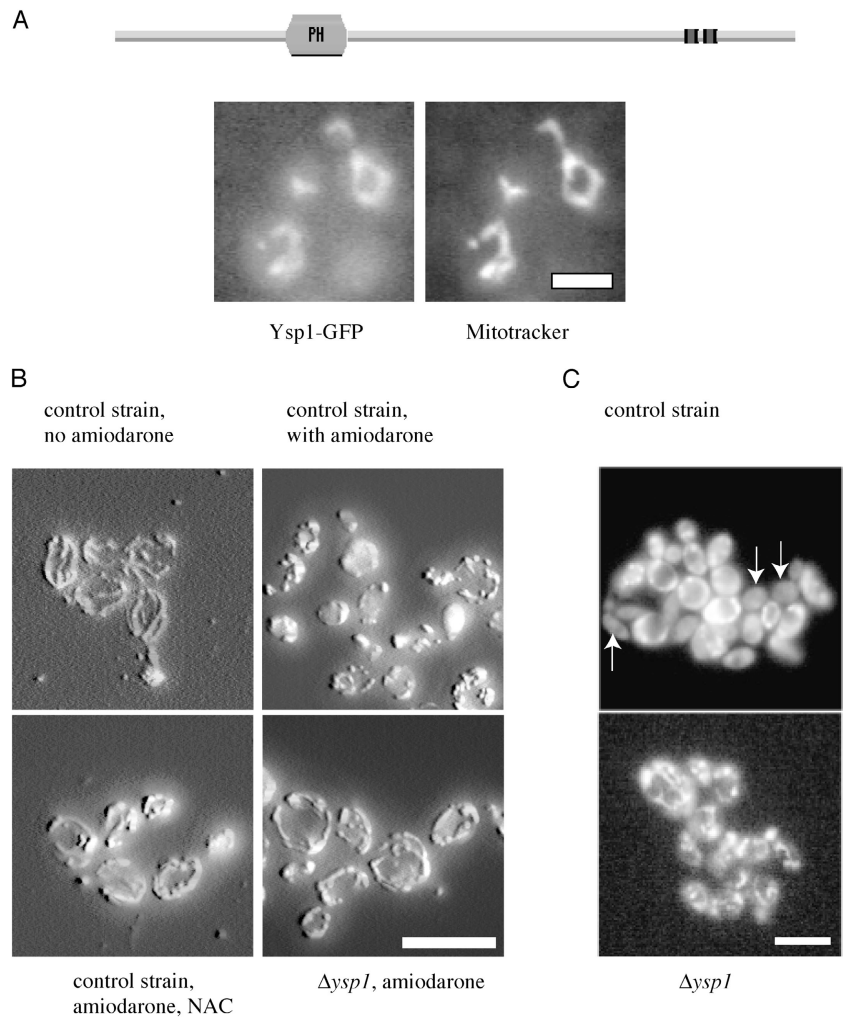## Amiodarone-induced Ysp1-dependent fragmentation of mitochondrial filaments

Fragmentation of mitochondrial filaments ("the thread-grain transition") is a typical marker of apoptosis in animals (for review see Skulachev et al., 2004). Recently it was shown that mitochondria fission is a necessary step in acetic acid– or H_2O_2-induced yeast cell death (Fannjiang et al., 2004). We have found that the same is true for our experimental system of yeast cell death. A genetic screen for proteins involved in the pheromone-induced cell death allowed us to identify a novel protein required for this process. To screen for genes involved in the cell death cascade, transposon-mutagenized yeast (Burns et al., 1994) were treated with high doses of the pheromone and the surviving cells were grown into colonies. We reasoned that the cell survival could be either due to (a) specific loss of a protein involved in the death cascade, (b) loss of sensitivity to the pheromone at a stage common to the mating and death cas-

cades (sterile phenotype), or (c) loss of functional mitochondria. To eliminate the latter two possibilities, we tested the ability of the surviving cells to mate with the cells of opposite sexual type and to grow on a nonfermentable carbon source (see the online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200408145/DC1).

We identified a gene, the deletion of which did not reduce the sensitivity of the mutant to low doses of the pheromone, did not prevent the mutant from growing on glycerol (not depicted), and significantly reduced cell death after the treatment with high pheromone concentration (Fig. 1 C). The gene in question (YHR 155W) proved to be an ORF with no clear homologies to genes of higher organisms. The protein sequence predicted the existence of a pleckstrin homology domain and two transmembrane domains (Fig. 7 A). We called the protein Ysp1 (yeast suicide protein 1). The large-scale screen for the localization of GFP-fusion proteins (Huh et al., 2003) has shown that Ysp1 is a mitochondrial protein. We confirmed that finding by imaging Mitotracker orange in the cells expressing Ysp1-GFP (Fig. 7 A). Similar images were obtained by immunofluorescent microscopy of yeast carrying Ysp1-Myc (unpublished data).

As is shown in Fig. 7 B, amiodarone causes fragmentation of mitochondrial filaments, the main mitochondrial structures in *S. cerevisiae* under the growth conditions used, to small spherical mitochondria. This process was strongly suppressed in the *ysp1* deletion mutant. In contrast, mitochondrial ΔΨ rise and the ROS level increase (Fig. 5 B) proved to be Ysp1 insensitive. Thus, Ysp1 seems to operate at the stage of the thread-grain transition rather than of the ΔΨ increase or the ROS elevation. In wild-type cells, ROS were essential for the

Figure 7. Ysp1localization and function. (A) Colocalization of the Mitotracker orange and Ysp1-GFP inside the yeast cell. Bar, 5 μm. In the top part of the figure, a domain structure of Ysp1 is shown; it contains predicted pleckstrin homology (PH) domain and two predicted transmembrane domains (on the scheme, in black). (B) Ysp1 is required for the thread-grain transition of mitochondrial filaments at a late stage of the amiodarone-induced suicide cascade. To image the mitochondrial network in the individual cells, 20 optical slices per field of view with 1.5-μm increments were collected and the resulting Z-stack was collapsed into a single image. Bar, 10 μm. (C) Ysp1 deletion prevents the amiodarone-triggered mitochondria de-energization. Cells with a dim diffuse Mitotracker orange staining (arrows) are present in the control strain but not in the mutant. Bar, 10 μm.

A

PH

Ysp1-GFP   Mitotracker

B

control strain,   control strain,
no amiodarone   with amiodarone

control strain,   Δysp1, amiodarone
amiodarone, NAC

C

control strain

Δysp1

tread-grain transition, because quenching of ROS by NAC prevented fragmentation of mitochondrial filaments (Fig. 7 B).

The stage of the apoptotic process in animal cells that follows the thread-grain transition may be the opening of the permeability transition pore in the inner mitochondrial membrane and, as a consequence, the collapse of $\Delta\Psi$ (Skulachev et al., 2004). We checked whether or not the loss of $\Delta\Psi$ also happens in our system. As shown in Fig. 7 C, at a late stage of the amiodarone-induced death, yeast cells appear that do not accumulate Mitotracker orange in their mitochondria (in this case, the $\Delta\Psi$ probe was added 15 min after amiodarone). Importantly, mitochondria de-energization did not occur in the $\Delta ysp1$ (Fig. 7 C). In the wild-type yeast, the effect proved to be arrested by antioxidants or oligomycin (unpublished data).

Thus, our data show that the thread-grain transition is a necessary event in the pheromone/amiodarone-induced cell death cascade occurring downstream of elevated $\Delta\Psi$-driven ROS formation, and Ysp1 is required for execution of the transition.

Discussion

The aforementioned data, together with our previous observations (Severin and Hyman, 2002), can be summarized by a scheme of the pheromone-induced programmed death shown in Fig. 8. The scheme assumes that a high pheromone level results in such a strong activation of the well known MAPK cascade involved in the pheromone-induced mating response (stages 2–5) that the Ca^{2+} concentration in cytosol appears to be above a threshold value required to trigger a specific chain of events in mitochondria (stages 6–10), eventually causing the cell death (stage 11). As for amiodarone, it operates in such a way that the cytosolic $[Ca^{2+}]$ increase arises with no MAPK cascade involved. High Ca^{2+} is assumed to activate mitochondria in two ways. It stimulates activity of certain respiratory enzymes and increases coupling of respiration and energy conservation. As a result, $\Delta\Psi$ increases and becomes the factor limiting the respiration rate. The latter conclusion is drawn from the fact that 1 μM FCCP stimulates and oligomycin inhibits respiration when amiodarone is added.

The rise of cytosolic $[Ca^{2+}]$ is the crucial event in the suggested scheme. Indeed, both pheromone (Ohsumi and Anraku, 1985) and amiodarone (Gupta et al., 2003; Courchesne and Ozturk, 2003) were shown to initiate a strong increase in the cytosolic $[Ca^{2+}]$. We confirmed these observations (Fig. 1 G). We also showed that $[Ca^{2+}]$ stimulates mitochondrial outer NADH dehydrogenase (the same was shown in plants; Ras-

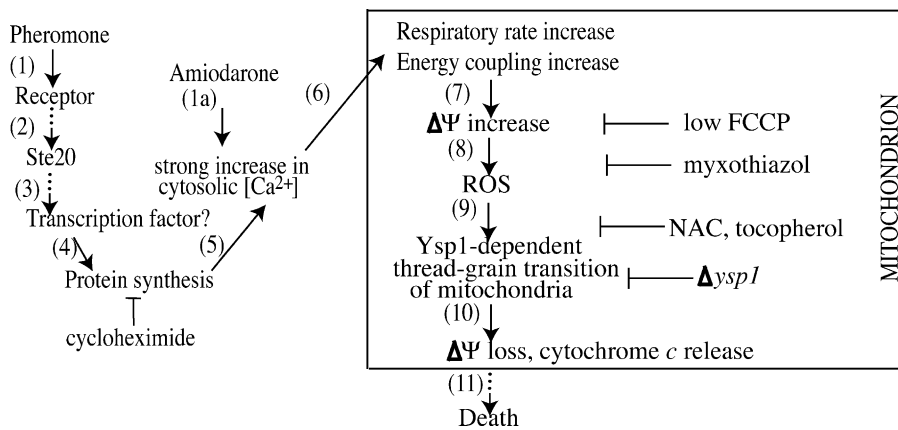Figure 8. **Proposed scheme of the programmed death cascade initiated by the pheromone and amiodarone.** See the text for explanations.

musson and Moller, 1991). This may explain the amiodarone-induced burst of respiration. As for an increase in energy coupling, Ca^{2+} might be competent in such an effect by inhibiting, e.g., the nonselective pore in yeast mitochondria (Perez-Vazquez et al., 2003). In our hands, Ca^{2+} ionophore A23187 induced mitochondria hyperpolarization and, in a fraction of cells, ROS accumulation and death.

Importantly, an increase in mitochondrial $\Delta\Psi$ was described to accompany several types of animal apoptosis (Banki et al., 1999; Li et al., 1999; Vander Heiden et al., 1999; Gottlieb et al., 2000; Sanchez-Alcazar et al., 2000; Scarlett et al., 2000; Matarrese et al., 2001; Piacentini et al., 2002; Gergely et al., 2002; Nagy et al., 2003). In yeast, such a phenomenon was shown to be inherent in the cell death induced by acetic acid (Ludovico et al., 2002) or by expression of Bax (Gross et al., 2000). In both of these cases, a $\Delta\Psi$ increase was followed by strong elevation of the ROS levels.

At stage 8 (Fig. 8), an elevation of $\Delta\Psi$ increases ROS production. In the intact respiratory chain of animal mitochondria, oxidizing succinate in stage 4, there are two major sites of $O_2^{\mp}$ generation: (1) the reverse electron transfer from $CoQH_2$ to O_2 via complex I and (2) reduction of O_2 by $CoQ^{\mp}$ in center o of complex III ($\sim$80% and 15% of ROS production, respectively; Korshunov et al., 1997). The former mechanism is absent in *S. cerevisiae* lacking complex I, but the complex III–linked $O_2^{\mp}$ generation is still possible (Fig. 9). Experimental data are in excellent agreement with the assumption that it is complex III that generates ROS in the *S. cerevisiae* mitochondria. First, a $\Delta\Psi$ rise caused by the amiodarone treatment resulted in stimulated ROS production, whereas simultaneous adding of FCCP entails both a $\Delta\Psi$ and ROS decrease (Fig. 5). Additionally, the myxothiazol inhibition of the Q cycle at stage of $CoQ^{\mp}$ formation (center o) decreased both $\Delta\Psi$ and ROS, whereas the antimycin A inhibition at stage of heme b_h oxidation (center i) decreased $\Delta\Psi$ and increased ROS (Fig. 6). Exactly these relationships were predicted by the Fig. 9 scheme and have been previously demonstrated on isolated rat heart mitochondria (Korshunov et al., 1997). Consistent with experiments on the intact cells, antimycin A strongly stimulated the H_2O_2 production by isolated yeast mitochondria.

Importantly, water- and lipid-soluble antioxidants (NAC and α-tocopherol, respectively) increased survival of yeast, in-

dicating that ROS are intermediates of the death program rather than its byproducts.

The scheme on Fig. 8 suggests that formation of ROS in mitochondria initiates the mitochondrial thread-grain transition and de-energization (Fig. 8, stage 9). Such a process was demonstrated in apoptotic animal mitochondria (for review see Skulachev et al., 2004). It proved to be a necessary step in various types of apoptosis (Frank et al., 2001), including yeast PCD induced by acetic acid or H_2O_2 (Fannjiang et al., 2004). Just at this step, a novel protein of the yeast programmed death cascade, Ysp1, seems to be involved. Ysp1 has no effect on respiration, $\Delta\Psi$, and ROS increases. However, decomposition of mitochondrial filaments did not occur in the $\Delta ysp1$ mutant (Fig. 7 B). In line with the aforementioned reasoning, an overexpression of Ysp1 under the GAL promoter induces fragmentation of mitochondrial filaments (unpublished data).

The final step of the mitochondria-related events (Fig. 8, stage 10) consists in their de-energization so that Mitotracker orange fails to stain any yeast cell organelles (Fig. 7 C). Again, this event is not observed in the $\Delta ysp1$ mutant. De-energization was accompanied by a release of cytochrome c from mitochondria to cytosol (Fig. 1 D).

One might speculate that the decomposition of mitochondrial filaments in yeast occurs by the same mechanism as in mammalians cells, i.e, by opening of a pore in the inner mitochondrial membrane. This opening is known to entail swelling of the matrix, disruption of the outer membrane, and a release of cytochrome c and other proapoptotic proteins from the mitochondrial intermembrane space to cytosol. In mammalian cells, it is an obligatory step of the mitochondria-mediated apoptosis (for review see Skulachev, 1996b). In yeast, Ludovico et al. (2002) have reported that the acetic acid–induced programmed death of *S. cerevisiae* is accompanied by release of cytochrome c from mitochondria to cytosol. Mutation in the cytochrome c heme lyase partially rescued the cells from death. Additionally, data obtained in our group (Severin and Hyman, 2002) show that a mutant lacking both c-type cytochromes survived in the presence of a lethal α-factor concentration in spite of the burst of ROS formation. The same effect was now observed with amiodarone. This might be interpreted as an indication to direct participation of cytochrome c in the yeast programmed death like that in animal apoptosis. Interestingly, the cascade of the

Figure 9. Q-cycle in the inner mitochondrial membrane of *S. cerevisiae*. (1) Reduction of CoQ by two electrons coming from a noncoupled NADH-CoQ reductase and heme b_H, followed by consumption of $2H^+$ from mitochondrial matrix. This occurs near the inner surface of the inner mitochondrial membrane (center *i*). (2) Diffusion of $CoQH_2$ from center *i* to center *o* localized near the outer membrane surface. (3) One-electron oxidation of $CoQH_2$ by the nonheme FeS cluster of Complex III (FeS_{III}). Here, reduced FeS_{III} and anion-radical $CoQ^{\mp}$ are formed. $2H^+$ are released to the intermembrane space. (3a) FeS_{III} is oxidized resulting in an electron transfer to O_2 via cytochromes c_1, *c*, and cytochrome oxidase, which forms H_2O. (4) $CoQ^{\mp}$ oxidation by heme b_L. (4a) Alternatively, $CoQ^{\mp}$ can be oxidized by O_2 in a $O_2^{\mp}$-generating fashion. (5) Transmembrane electron transfer from b_L to b_H. (6) The CoQ diffusion back to center *i*. Operation of Q-cycle results in generation of $\Delta\Psi$ and ΔpH (matrix being negatively charged and alkalinized). $\Delta\Psi$ inhibits the $b_L \rightarrow b_H$ electron transfer and, hence, stimulates alternative $CoQ^{\mp}$ oxidation by O_2 and generation of $O_2^{\mp}$. Uncoupler discharges $\Delta\Psi$ by passive H^+ influx from the intermembrane space to matrix and, as a result, inhibits the ROS generation. Myxothiazol (myxo) also suppresses ROS generation by preventing $CoQH_2$ oxidation to $CoQ^{\mp}$ in center *o*. As for antimycin A (anti), it stimulates ROS production by inhibiting oxidation of b_H and, as a consequence, of b_L.
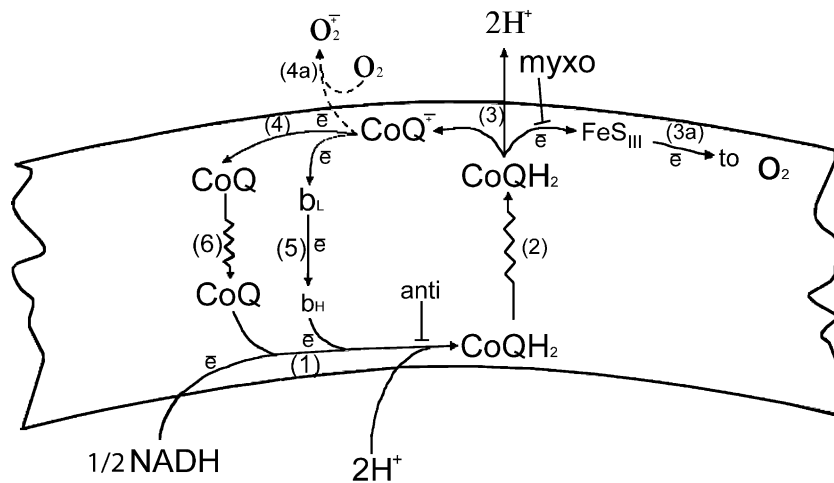
pheromone-induced programmed death of yeast resembles to some degree that initiated by tumor necrosis factor α in HeLa cells or by Fas-ligand in T cells. In the latter case, an extracellular ligand combines with a receptor on the plasma membrane and activates MAPK cascade leading to mitochondrial hyperpolarization and ROS production (Nagy et al., 2003). Similar to α-factor, Fas-ligand–induced activation of the MAPK cascade can either lead to cell proliferation or cell death, depending on the ligand amount (for review see Nagy et al., 2003).

One difference between the two models is that in yeast PCD protein synthesis is required, whereas it is not necessary as a rule for mammalian apoptosis. Apparently, in multicellular organisms, the suicide apparatus is usually ready to be activated, whereas, in unicellular ones, it requires de novo production of some proteins. These proteins are involved upstream of the amiodarone-sensitive step because the cell killing by this agent is protein synthesis independent.

Another difference between the two models is that although in mammalian cells mitochondria are always active in energy production, in glycolyzing yeast mitochondria "switch on" only during apoptosis. Our data show that the switching on occurs by both the activation of respiration and an increase in energy coupling. Probably the existence of the Ca^{2+}-activated NADH dehydrogenase and Ca^{2+}-inhibited, nonselective pore in yeast mitochondria and their absence in mammals partly explains this difference between the two models. Remarkably, the suicide of aging yeast cells is, like that of the pheromone- or amiodarone-treated ones, accompanied by activation of respiration (Jazwinski, 2002). Apparently, the yeast life strategy dictates that yeast cells, to obtain energy, use glycolysis if sugars are in excess. As for respiration, it is low and essentially uncoupled, being apparently used in some nonenergetic functions like scavenging of glycolytic products, decrease of intercellular $[O_2]$, etc. Such a modus vivendi allows cells to synthesize ATP and at the same time to minimize the "oxygen danger." In contrast, when a suicide program is initiated, respiration becomes tightly coupled and its maximal electron transfer capacity strongly increases to produce ROS at high rate in the middle part of the respiratory chain (Fig. 9, Q-cycle).

The programmed death looks quite reasonable if we consider malfunctioning cells of a multicellular organism. As for an entire organism, the possibility of the existence of a death program has been discussed since the second half of the nineteenth century when Alfred Russell Wallace and August Weismann put forward such an idea (Weismann, 1889). Now, programmed death is documented in unicellular organisms (i.e., bacteria [for reviews see Ameisen, 1996; Raff, 1998; Lewis, 2000; Engelberg-Kulka et al., 2004] and yeast [for reviews see Jin and Reed, 2002; Madeo et al., 2002a; Skulachev, 2002; Weinberger et al., 2003; Breitenbach et al., 2003; Fabrizio et al., 2004] and some multicellular organisms (Skulachev, 2003). To distinguish the organismal biochemical suicide from similar events occurring at suborganismal levels, the programmed death of an organism was defined as "phenoptosis" (Skulachev, 1999). Its biological significance for yeast undergoing sexual reproduction could be in the killing of cells that fail to mate. Perhaps, high pheromone concentrations appearing in clefts between adhered haploid cells kill these cells if they cannot, for a long time, convert to the pheromone-resistant diploids (Severin and Hyman, 2002; Skulachev, 2002). Moreover, even if the suicide mechanism does not discriminate the "weak" and the "strong" cells, it still might be useful for the community: random elimination of organisms could accelerate the change of generations and thus the finding of new, useful traits (Weismann, 1889).

Materials and methods

Strains and growth conditions

The strains used in this study, namely, TH40 (W303, Mat *a*), TH923 (*cmd1-66*; original strain provided by T. Davis, University of Washington, Seattle, WA), TH927 (*rho*[0]), TH1003 (*cyc3::URA33*; original strain provided by F. Sherman, School of Medicine and Dentistry, University of Rochester, Rochester, NY), FS1 (*ysp1::HIS*), FS5 (*YSP1-GFP::*

HIS), and FS6 (*GAL::TRP, YSP1-GFP::HIS,*) are all derivatives of W303. Yeast extract with peptone (Rose et al., 1990) was supplemented with either 2% glucose (α-factor experiments) or either 2% raffinose or 2% galactose and 2% raffinose (amiodarone experiments). Yeasts were typically grown to the density 2×10^6 cells/ml before the α-factor or amiodarone treatment.

Assays for ΔΨ, ROS, cell survival, and intracellular Ca²⁺

Mitochondrial ΔΨ was visualized with Mitotracker orange (0.1 μg/ml; Molecular Probes). Intracellular ROS were revealed with 50 μM dichlorofluorescin diacetate (H₂DCF-DA; Sigma-Aldrich).

Cell survival for amiodarone experiments was determined by incubating the cells at 30°C in the shaker, followed by plating an aliquot on solid media and counting the colonies the next day. The percentage of cell survival was calculated based on counting the cells under the microscope before plating. Mitotracker orange staining was photographed after 10–15 min, and H₂DCF-DA after 20–30 min following amiodarone addition. Photographs were taken at RT with a camera (model C4742-95; Hamamatsu) mounted on an Axioplan 2 (Carl Zeiss MicroImaging, Inc.). A 63× objective lens was used. At least three experiments were performed for each treatment. In the α-factor experiments, the dead cells were visualized by Methylene blue (Sigma-Aldrich) or propidium iodide.

Immunofluorescent staining was performed as described in Piatti et al. (1996) using a 1:200 dilution of anti–cytochrome *c* antibody (clone 6H2.B4; BD Biosciences).

For measurements of intracellular Ca²⁺, cells were loaded overnight with 3 μM Fluo-3/AM (Sigma-Aldrich), diluted to OD 0.5 the next morning in fresh media containing the same concentration of the dye, and shaken for 2–3 h. Cells were washed twice in water, A23187 (50 μM, Sigma-Aldrich), amiodarone, or buffer controls were added and the cells were photographed under the microscope.

Total yeast DNA was prepared as described previously (Koch et al., 1996) with modifications (see online supplemental material).

TUNEL

TUNEL was performed using in situ Cell Death Detection Kit and TMR red (Roche Diagnostics GmbH). Cells were fixed and digested with zymolase as described in Piatti et al. (1996), with the exception that the zymolase treatment was performed for 2 h at 37°C. Triton X-100 was added to 0.2% for 5 min, protoplasts were spun at 5,000 rpm for 5 min, resuspended in 50 μl of TUNEL labeling mix, and incubated at 37°C for 1 h. Then, the protoplasts were washed twice and finally resuspended in 20 μl of PBS, Hoechst dye was added to 1 μg/ml to visualize DNA, and the protoplasts were analyzed by microscopy.

Respiration and H₂O₂ generation

The cell or mitochondrial respiration was measured using standard polarographic techniques with a Clark-type electrode. Incubation medium for cells contained 50 mM KH₂PO₄, pH 5.5, and 0.05% glucose. In some experiments, the YPGalRaf medium was used. Incubation medium for mitochondria contained 0.6 M mannitol, 10 mM Tris-HCl, 2 mM potassium phosphate, pH 7.4, 4 mM pyruvate, and 1 mM malate. Mitochondria were prepared essentially as described previously (Bazhenova et al., 1998).

H₂O₂ generation was measured fluorimetrically by means of HRP and Amplex red. Incubation mixture contained 0.6 M mannitol, 10 mM Tris-HCl, 2 mM potassium phosphate, pH 7.4, 1.2 μM Amplex red, and 0.05 mg/ml peroxidase.

Online supplemental material

The online material presents the description of the screen for genes involved in the pheromone-induced cell death cascade and our protocol for total yeast DNA purification. The online supplemental material is available at http://www.jcb.org/cgi/content/jcb.200408145/DC1.

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